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IMPROVING TREATMENT OUTCOMES FOR PATIENTS WITH PULMONARY TUBERCULOSIS IN TANZANIA:
Host and Pathogen factors

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Improving treatment outcomes for patients with pulmonary tuberculosis in Tanzania: Host and Pathogen factors

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"Life is like riding a bicycle. To keep your balance, you must keep moving."

Albert Einstein

To the loving memory of my father

ABSTRACT

Tuberculosis (TB) causes more than 1.2 million deaths each year globally. Yet, survivors of tuberculosis are left with long term sequelae of chronic inflammatory responses with consequent reduced quality of life. Biomarkers of monitoring tuberculosis disease activity, clinical response and mortality might assist in reducing mortality and ultimately in improving quality of life post tuberculosis disease.

The general aim of the thesis was to describe mortality among patients treated for a first episode of tuberculosis in Tanzania in relation to selected known pathogen related predictors of poor tuberculosis outcomes, namely HIV and anti-tuberculosis drug resistance. We also explored the role of cytokines as part of the host immune response, in relation to mortality and in modulating lung damage.

In Paper I: We show an overall mortality of 3.4 – 9.3% among patients treated for tuberculosis in Tanzania, and that TB/HIV patients were at a higher risk of death compared to TB mono-infected patients. The best benefits of antiretroviral therapy (ART) in reducing mortality for TB/HIV co-infected patients occurred when ART was initiated after 14 days of anti-tuberculosis therapy (ATT). We also observed **(Paper II)** that among 861 patients with tuberculosis, those with isoniazid resistance without concomitant rifampicin resistance had an increased risk of unsuccessful treatment outcome (death or treatment failure or loss from follow up - combined). In **Paper III**, we observed that patients who exhibited, in cell-based assays, higher interferon gamma (IFN-γ) responses against cytomegalovirus (CMV), Epstein Barr virus (EBV) or *Mycobacterium tuberculosis* ESAT-6 antigens at the time of TB diagnosis had a survival benefit following treatment for TB. However, IFN- γ responses to some viral antigens (H5N1 and HSV-1) as well as other mycobacterial antigens (Ag85A, Rv2958c, Rv0447c) were not significantly different between patients who survived and those who died. In **Paper IV**, we observed very high levels of interleukin 6 (IL-6) in serum from 234 patients with pulmonary tuberculosis, as compared to levels of IL-6 in serum from seven healthy controls. Other cytokines (IFN-γ, TNF-α, IL-2, IL-10, IL-17A and IL-21) were also analyzed at the time of TB diagnosis. Unlike with the other cytokines which returned to pretreatment levels or below following ATT, IL-6 levels at end of ATT were significantly higher than their corresponding pre-treatment levels. In addition, we also found that higher IL-6 levels at TB diagnosis correlated with survival of patients with pulmonary tuberculosis, as well as with severe lung injury, defined by chest x-ray score more than 80 at diagnosis.

In conclusion, between 3 and 9 out of 100 Tanzanian patients treated for first time tuberculosis will die during treatment. Well planned ART, appropriate clinical monitoring and timely addressing of background isoniazid resistance are essential to improve treatment outcomes. While anti CMV and EBV immune responses may serve to stratify mortality risk, adjunct therapy with IL-6 may serve as a possible target in reducing lung damage and consequently aid to improve quality of life following *Mycobacterium tuberculosis* disease in the future.

LIST OF SCIENTIFIC PAPERS

I. **Nagu TJ**, Aboud S, Mwiru R, Matee MI, Rao M, Fawzi WW, Zumla A, Maeurer MJ, Mugusi F. Tuberculosis associated mortality in a prospective cohort in Sub Saharan Africa: Association with HIV and antiretroviral therapy. **Int J Infect Dis. 2017; 56:39-44.**

II. **Nagu TJ**, Aboud S, Matee MI, Maeurer MJ, Fawzi WW, Mugusi F. Effects of isoniazid resistance on TB treatment outcomes under programmatic conditions in a high-TB and -HIV setting: a prospective multicentre study. **J Antimicrob Chemother. 2017;72(3):876-881.**

III. **Nagu T,** Aboud S, Rao M, Matee M, Axelsson R, Valentini D, Mugusi F, Zumla A, Maeurer M. Strong anti-Epstein Barr virus (EBV) or cytomegalovirus (CMV) cellular immune responses predict survival and a favourable response to anti-tuberculosis therapy. **Int J Infect Dis. 2017;56:136-139**

IV. **Nagu TJ**, Rao M, Axelsson-Robertson R, Aboud S, Matee M, Fundikira LS, Nkumbih ZF, Poiret T, Valentini D, Mugusi F, Zumla A, Maeurer M.
Cytokine Biomarkers in Tanzanian patients with Pulmonary TB - a prospective longitudinal cohort study. **(Manuscript)**

CONTENTS

LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cells
ART	Antiretroviral therapy
ATT	Anti-Tuberculosis Therapy
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
CT	Computerized Tomography
CTC	Care and Treatment Centers
CXR	Chest X-Ray
DOT	Direct Observed Therapy
DST	Drug Susceptibility Test
E	Ethambutol
ELISA	Enzyme-Linked Immunosorbent Assay
EPTB	Extra Pulmonary Tuberculosis
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IGRAS	Interferon Gamma Release Assays
IL-1β	Interleukin 1 beta
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-2	Interleukin 2
IL-21	Interleukin 21
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
INH	Isoniazid
IPT	Isoniazid Preventive Therapy
LJ	Löwenstein–Jensen
LPA	Line Probe Assay
LTBI	Latent tuberculosis infection

M.tb	*Mycobacteria tuberculosis*
MDR	Multi Drug Resistance
MGIT	Mycobacteria Growth Indicator Tube
MNH	Muhimbili National Hospital
MRI	Magnetic Resonance Imaging
MUHAS	Muhimbili University of Health and Allied Sciences
NAAT	Nucleic Acid Amplification Test
NHP	Non-Human Primates
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NTLP	National Tuberculosis and Leprosy Program
OP	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD1	Program cell Death 1
PET	Positron Emission Tomography
PTB	Pulmonary Tuberculosis
RIF	Rifampicin
SIDA	Swedish International Development Agency
SSA	Sub Saharan Africa
TB	Tuberculosis
Th1	T helper 1
TIM3	T cell Immunoglobulin and Mucin domain 3
TNFα	Tumor Necrosis Factor - alpha
TST	Tuberculin Skin Test
WBA	Whole Blood Assay
WHO	World Health Organization
XDR	Extensive Drug Resistant
YLD	Years Lived with Disability
Z	Pyrazinamide
ZN	Ziehl Neelsen

1 INTRODUCTION

"…She is my only child and a mother of a 5 year old child. ***Will she survive or die?*** *I need her, for I have nothing to offer to the child…."*A voice of a crying woman very early on a Monday morning keeps echoing in my mind as one of the very many cases physicians in Tanzania encounter. This is a vivid image of many years from which I find reason, enthusiasm and strength to carry on with research in infectious diseases - particularly, tuberculosis (TB) and human immunodeficiency virus (HIV) infection. The question posed by the sorrowful mother is a difficult one, which unequivocally demands an answer. At this juncture, we are able to provide patient prognosis largely based on clinical and epidemiological evidence – which is associated with a high level of credibility. Nevertheless, enough patients succumb to TB even in the absence of known predictors of bad outcomes such as HIV co-infection and/or multidrug resistance. In Tanzania, routinely collected TB reports show that 90% of patients newly treated for TB in Tanzania will be cured while about 6% will die during therapy [1]. Every life counts – therefore, there is an urgent need to describe and characterize factors that could provide clues to so many unanswered questions in clinical TB. These answers may also aid in reducing mortality by addressing hitherto unknown factors – so we may one day say to such a grieving mother with great certainty that no one dies of TB or at least, very rarely in our setting! If patients with TB survive, the final outcome may not necessarily be all too positive; 74% of them will develop clinical features of chronic lung disease in the last month of their TB treatment [2]. Yet, in HIV-infected individuals, a previous episode of TB increases the risk of another TB episode and death [3, 4]. Healthcare researchers and practitioners therefore have the obligation to learn how to minimize both the death toll as well as lung damage in patients with TB. In this thesis, we explore factors that could be linked with survival and lung damage in patients with TB, paving the way in search of better tools to improve survival as well as quality of life for patients with TB in Tanzania and internationally.

1.1 TUBERCULOSIS

1.1.1 Etiology and pathogenesis

Tuberculosis (TB) is a deadly disease affecting millions of people, and is caused by the bacterial pathogen *Mycobacterium tuberculosis* (*M.tb*), which was first described by Robert Koch in 1882 [5]. The infection is transmitted through aerosol droplets from an infected individual by coughing, sneezing, singing or mere talking [6-8]. However, it appears that cough is most effective in transmitting the bacteria, not only due to the number of infectious particles expelled, but largely due to the small size of the droplets, which are persistently suspended in air [6-8]. Transmission risk is affected by contagiousness of the source (infected person), usually determined by the *M.tb* load in sputum and the proximity between the source and other individuals [9]. The extent of TB disease in the lung - which in turn increases the frequency of coughing – also greatly affects transmission dynamics, alongside the afore-mentioned factors [8, 9]. In this regard, public health strategies to reducing TB must continue to

address improved living conditions and early case detection and treatment [10]. Limiting the extent of pulmonary TB disease by reducing host lung tissue damage using adjunct therapies might also be mutually beneficial to achieve a reduction in lung damage and therefore transmission dynamics.

Inhaled *M.tb* droplets will be transported to the respiratory bronchioles where they will be phagocytosed by alveolar macrophages residing in the lower and middle lung zones [11-13]. Effective containment of *M.tb* by the host's immune system establishes a latent infection, termed as latent TB infection (LTBI) as opposed to *M.tb* propagation in the host leading to clinical disease (active TB). Therefore, deficiency in innate immune function [14] and/or adaptive immunity heavily compromises control of *M.tb* infection, fosters infection transmission, and increasing possibilities to succumbing to the disease [15]. Acquired immunodeficiency due to HIV co-infection is a major contributor to TB-related deaths, and presents an important clinical setting to study immune cell interactions in TB disease [16, 17].

The hallmark of TB pathogenesis lays in the initiation; maturation and maintenance of granuloma as a result of the interplay between innate and adaptive immunity [18]. Neutrophils are engaged very early on. Activated by *M.tb* products, neutrophils are responsible for the recruitment of leucocytes and promotion of the inflammatory process. T helper 1 (Th-1) response of the adaptive immunity is central to the formation and maturation of the granuloma [19]. The typical TB granuloma, as seen in figure 1, has central caseous necrosis surrounded by different types of macrophages; (epithelioid, multinucleated - Langhans, foamy) all encircled with a layer of lymphocytes [18, 19]. In non-human primates (NHP) TB granuloma formation has been shown to begin at the hilar lymph nodes after three weeks of infection thereafter involving the regional thoracic lymph nodes and subsequently the lungs approximately four to six weeks following infection [20].

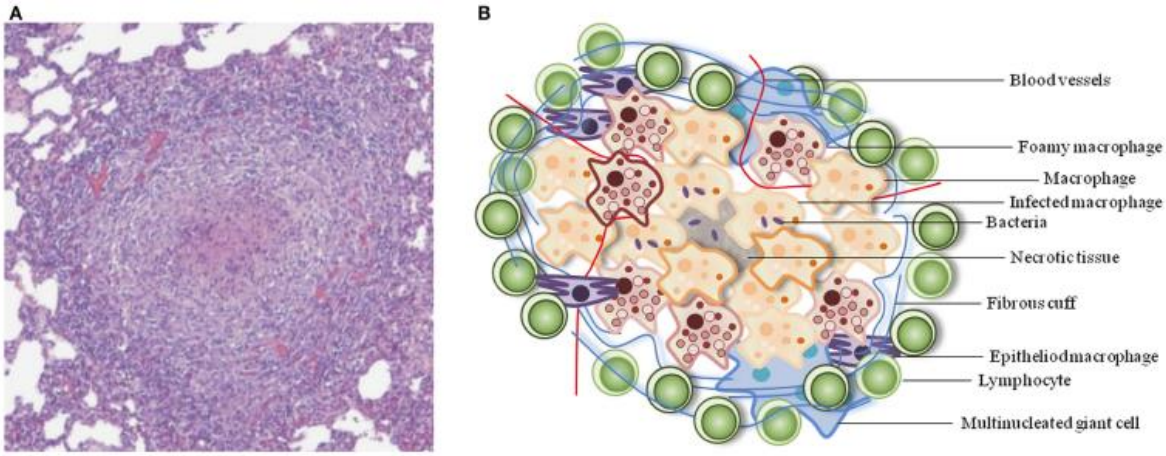


Figure 1: A. Granuloma architecture from tissue histological section (Source: Gil el al PlosOne 2010) B. Schematic cellular representation of TB granuloma (Source: Guiradoe et al 2013) both images are taken from reference [18]

Disruption of the granuloma results into cavity formation in lungs. Consequently, TB lesions observed is a mixture of inflammatory and tissue healing processes characterized by mixed

immuno-physiological features such as; consolidation, calcification, cavity formation and fibrosis, as well as immune cell infiltrates comprising lymphocytes and myelocytic cells [20]. Although TB primarily affects the lungs, *M.tb* can disseminate through the lymphatics to the rest of the body [20, 21].

1.1.2 Global epidemiology of tuberculosis

It is estimated that about 1.7 – 1.9 billion people worldwide harbour LTBI [22-24]. The recent global burden of disease estimates that latent tuberculosis is the second most prevalent infection globally, responsible for 1 out of 4 years lived with disability (YLD) in 2016 [24]. In general, 10% of individuals with LTBI will develop TB disease at some point in their lifetime [25]; the risk is higher in HIV co-infected persons and other in immunosuppressive scenarios such as cancer, transplantation and diabetes [26, 27]. Males carry a disproportionately larger burden of TB disease, and are presumptively the source for propagating the infection [28, 29].

According to global statistics, it is estimated that in 2015 there were 10.4 million new patients who developed TB [23]. More than 80% of these cases are in 30 countries classified as high burden TB countries, mainly in Sub-Saharan Africa (including Tanzania) and Eastern Europe, South East Asia and China [23]. The estimated global TB burden is represented on figure 2 [23].

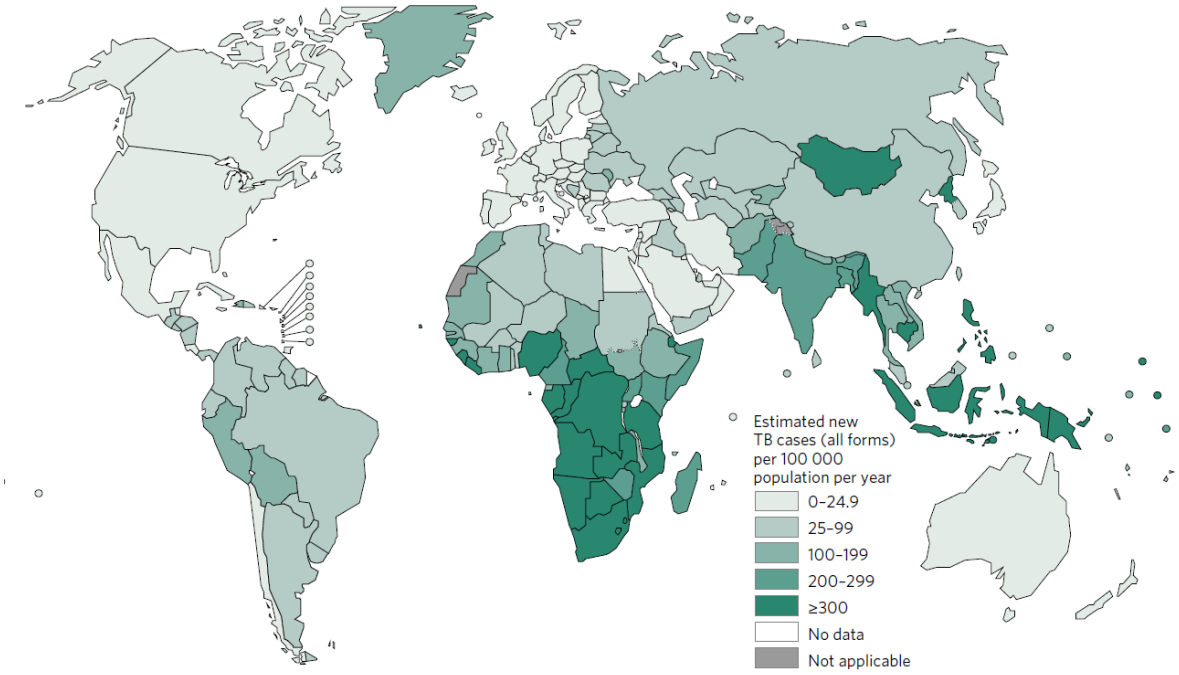


Figure 2: Esimated global tuberculosis incidence rate 2015 (Source: The WHO - global tuberculosis report 2016)[23]

Drug-resistant TB, largely comprising multidrug resistance (MDR-TB) and extensive drug resistance (XDR-TB) in addition to HIV co-infection are the major drivers of the global TB epidemic as well as severe TB disease and increased mortality [23, 24, 30]. MDR-TB is defined as resistance to at least isoniazid (INH) and rifampicin (RIF) [31], while XDR-TB is defined as resistance to INH, RIF and any fluoroquinolone i.e. moxifloxacin, levofloxacin, gatifloxacin and at least one of three injectable second-line drugs i.e. kanamycin, capreomycin and amikacin [31]. The global prevalence of MDR-TB is estimated to be 3.3 – 3.9 % [23, 32], mainly centred in the South East Asia region as well as China [23].

Re-occurrence of the TB epidemic coincided with the emerging of HIV epidemic in the early -1990s [26]. Globally, there were approximately 0.5 million HIV-infected individuals with TB reported by the World Health Organisation (WHO) in 2016, while circa 15% of notified TB patients who underwent HIV testing had HIV co-infection [23]. As depicted in figure 3, the prevalence of HIV among patients with TB has the highest impact in Sub-Saharan Africa (SSA) where about 80% of TB/HIV cases are usually reported [23]. In 2015, the prevalence of TB/HIV co-infection was 36% in the SSA region, particularly high in South Africa [23, 24]. Only 58% of the estimated MDR-TB cases are treated, while only 55% of all reported TB cases in 2015 had an HIV test with even fewer patients receiving Antiretroviral Therapy (ART) [23]. Missed opportunities for treatment such as these should be minimised as we get closer to ending TB by 2030 [33].

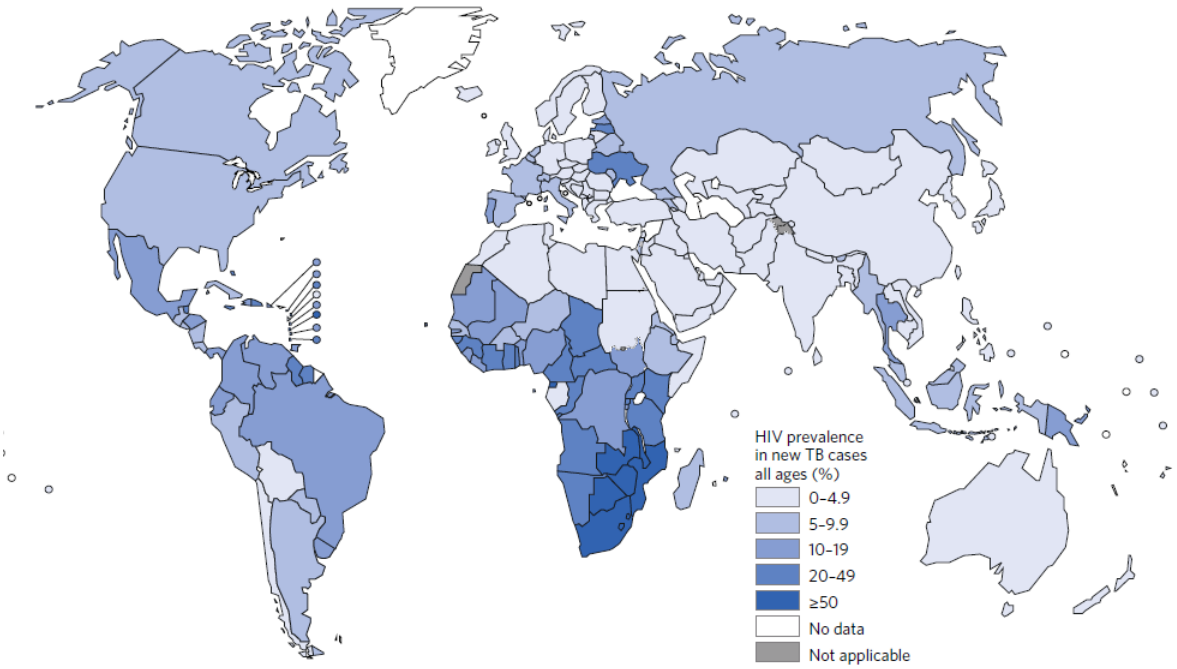


Figure 3: Estimated HIV prevalence among patients with tuberculosis in 2015 (Source: The WHO - Global tuberculosis report 2016)[23]

In 2015, a total of 1.2 million deaths were due to TB; the vast majority had drug-sensitive TB (1.1 Million) while MDR/XDR-TB) contributed to a further 0.1 million deaths [30]. Figure 4 shows the estimated global number of incident TB cases and mortality trends from 2000 to

2015 [23]. Multiple efforts from different stakeholders have resulted in a reduction of TB cases as well as mortality among tuberculosis patients (figure 4). However, the WHO estimates the trend of decline is inadequate to attain the anticipated goals to end tuberculosis by 2030[23].

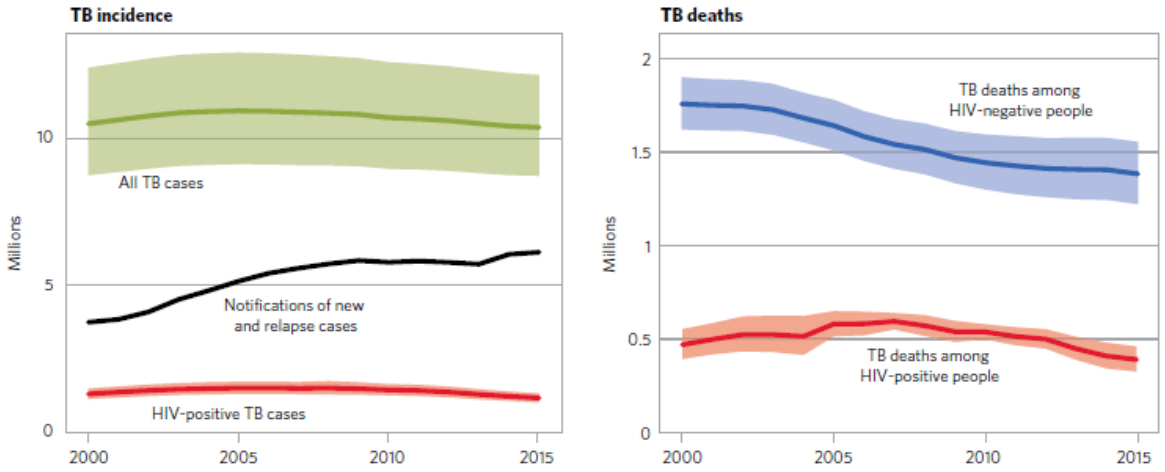


Figure 4: Global trends of estimated incident TB cases and mortality 2000 – 2015 (Source: The WHO – Global TB report 2016)[23]

1.1.3 Clinical manifestation of tuberculosis

Predominantly a pulmonary disease (pulmonary TB, PTB) in adults, TB has the potential to spread to and affect any part of the body (extra-pulmonary TB, EPTB), and is therefore a systemic disease [31, 34]. Clinical presentation of patients with TB is variable depending on the affected organ and immune response to the infection. In general, symptoms in PTB include cough, sputum production, low grade fever, night sweats, weight loss, difficulty in breathing and haemoptysis [35, 36]. These symptoms are non-specific and can be present in many other diseases. On the other hand, it is not uncommon to find disseminated TB presenting with non-classical features or mere asymptomatic among immuno-compromised individuals [37]. Therefore, in addition to clinical features, accurate and easy-to-use points of care TB diagnostics are highly necessary [36, 38].

1.1.4 Diagnosis of tuberculosis

TB diagnosis requires confirmation of the presence of *M.tb*. This can be achieved through bacteriological methods or molecular techniques such as Nucleic Acid Amplification Tests (NAAT) [39]. Conventional acid fast bacilli (AFB) staining on smear microscopy is a commonly used methods in the clinics in resource limited setting. *M.tb* culture on liquid or solid media is the current gold standard for TB diagnosis [39]. Molecular methods such as line probe assays (LPA) or Gene Xpert MTB/RIF are instrumental in identifying both TB and drug resistance patterns for clinical decisions [39-42]. The Gene Xpert MTB/RIF is an automated, real time Polymerase Chain Reaction (PCR) test that has revolutionized the diagnosis of TB and RIF resistance, thus recommended as standard of care in the context of high TB and HIV [38, 43].

Interferon gamma release assays (IGRAs) have been used for diagnosis of LTBI in several countries but are not able to distinguish between active disease and latent infection [44]. Thus they may not be so useful in countries with high TB burden [44]. Another challenge with IGRAs is the high variability between tests, making them rather difficult to use for diagnosis and/or monitoring of TB in high burden settings [45, 46].

Radio imaging techniques especially X-ray have been used in the evaluation of patients with TB both to assist in diagnosis, gauging the extent of tissue damage and monitoring treatment progress by assessing tissue healing [34]. There are various imaging features of PTB including combinations of consolidation, fibrosis, calcification, cavity, nodulation or fibrosis [47]. These features are not pathognomonic; there are other disease/pathologies such as sarcoidosis, lung adenocarcinomas and chronic granulomatous disease that mimic TB features on radio-imaging[48-50]. However, in settings of smear-negative PTB or TB osteomyelitis, plain radiographs have been useful [36, 48]. Unfortunately until recently, there were no simple, validated tools that allowed objective comparison of radiological images between TB patients as well as same patient at different time points during treatment [47].

Other imaging methods such as magnetic resonance imaging and computed tomography (CT-scan) with or without positron emission tomography (PET) are essential for the diagnosis of EPTB [51-53]. PET-CT and PET Magnetic Resonance Imaging (MRI) are increasingly used in clinical settings especially in TB of the bone and mimics of solid tumours [51, 54] but have also been used in research settings as for diagnostic evaluation and/or clinical assessment of cure[50, 55].

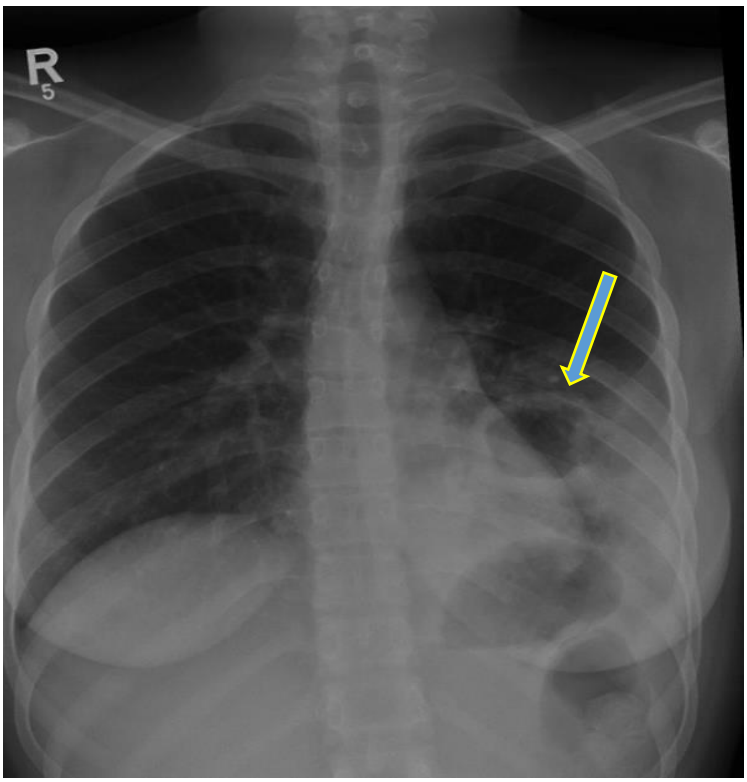


Figure 5: Chest x-ray of patient with pulmonary tuberculosis
(Case courtesy of Dr Hani Salam, Radiopaedia.org, rID: 12437)

1.1.5 Tuberculosis treatment

Assessment of anti-TB drug resistance prior to initiating treatment is essential for appropriate management. Treatment of drug-sensitive TB takes approximately six months, and comprises 5mg/kg isoniazid (INH), 10mg/kg rifampicin (RIF), 25mg/kg ethambutol (E) and 15mg/kg pyrazinamide (Z) for 2-3 months followed with 5mg/kg isoniazid and 10mg/kg rifampicin for the remaining time [56]. For re-treatment, patients are given a daily injection of streptomycin (15mg/kg injection) in addition to RIF, INH, Z, and E; for 2-3 months, depending on sputum conversion. It is therefore important to preserve the potency of isoniazid and rifampicin, which form the cornerstone in the treatment of drug-sensitive TB. Although treatment success rate globally is usually above 85% in most cases, there is a need to accelerate the development of new TB drugs to cater for *M.tb* drug resistance strains.

The treatment of MDR-TB is complex, requiring the use of many drugs which are less potent and often accompanied with unpleasant side effects [42, 57, 58]. The conventional treatment duration for MDR-TB is a minimum of 20 months as guided by *M.tb* culture conversion [42]. Six to seven out of ten patients with MDR-TB will be treated successfully after 20-24 months[23, 59]. The need for shorter and more effective treatments regimen led to the introduction and inclusion of the new anti-TB drugs Delamanid or Bedaquiline to TB drug regimens [42], which aim to achieve successful MDR-TB treatment in 9 – 12 months. Both drugs have shown faster and higher culture conversion rates, albeit with associated side effects e.g. arrhythmia. About 80 - 97% of patients with MDR – TB achieve negative cultures, within six months of treatment with Delamanid or Bedaquiline [60, 61].

1.2 CYTOKINES PLAY KEY ROLE IN MEDIATING IMMUNE RESPONSE TO *M. tuberculosis*

The success of *M.tb* to establish infection depends on its ability to multiply within the alveolar macrophages and disseminate by counter-acting the host response to control its propagation[11, 12, 20, 62]. Cytokines are soluble substances that mediate the cross talk between immune cells as well as between non-immune cells, and play an important role in relation to host control of TB through enhanced macrophage as well as lymphocyte activity and granuloma formation [14, 63] Many cytokines have been studied in relation to *M.tb* control; this interplay is complex and not completely understood, involving both the innate and adaptive immune cells, as depicted in figure 6 [14, 63, 64]. Several key cytokines have a primary role in *M.tb* pathogenesis and control: interleukin 12 (IL-12), IL-18 interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), IL-1β, IL-6, IL-17 and IL-2, as observed in animal models of TB and very importantly in patients with TB [62, 65, 66].

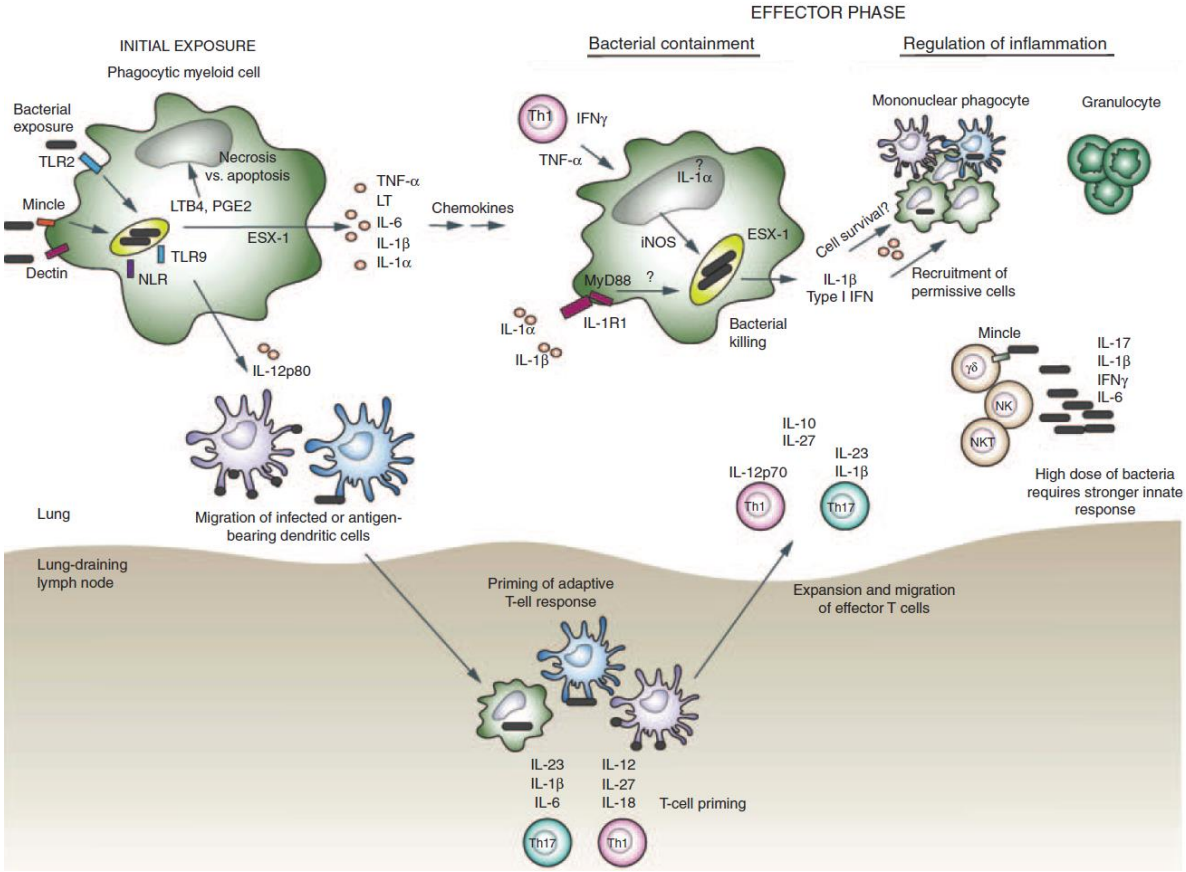


Figure 6: Interaction of the innate and T helper 1 cytokines following exposure with *Mycobacteria tuberculosis* infection (Source: Cooper Mucosal Immunol, 2011)[14]

The interaction between *M.tb* and macrophages induces a series of events leading to production of IL-12 and IL-18 [67-70]. IL-12 is important for the activation of immature dendritic cells as antigen presenting cells (APCs), which will be prompted to translocate from the site of infection to the regional lymph nodes to present *M.tb* antigens to T cells, priming of the adaptive immune response [66]. Priming of CD4+ T helper cell response follows antigen presentation in mediastinal lymph nodes about 10-14 days after the infection [20, 71]. Cytokines resulting from T helper 1 (Th1) pro-inflammatory responses, IFN-γ, TNF-α, IL-2 and possibly IL-17, in some cellular subsets are crucial in host defence against *M.tb* [66, 72-74].

IFN-γ is produced by activated CD4+ and CD8+ T cells and natural killer (NK) cells, and is important to enhance intracellular killing of *M.tb* by macrophages and dendritic cells – which also leads to antigen processing, presentation and augmentation of T-cell activation [75]. Deficiencies in the IFN-γ pathway are associated with failure to control *M.tb* infection, exaggerated tissue necrosis and death [15, 75, 76]. IFN-γ responses to specific pathogenic *M.tb* antigens has been utilised in the clinical diagnosis of LTBI in low incidence settings. However, its clinical utility as a diagnostic tool for active TB or gauge clinical response remains to be determined [46, 77].

During *M.tb* infection, TNF-α is produced initially by monocyte-derived cells, and later by T and NK cells following activation [65, 78]. Together with IFN-γ, TNF-α potentiates macrophage

phagocytic activity to engulf *M.tb* bacilli and promote apoptosis, which also amplifies the adaptive immune response signals [79]. TNF-α is also important in promoting neutrophil aggregation and is essential for the formation and maintenance of effective granulomas [65]. A fine-tuned balance of TNF-α function is important in modulating TB pathology. Higher levels of TNF-α have been shown to correlate with severe lung pathology [80, 81]. Alternatively, TNF-α blockade perpetrated increase in *M.tb* load, severe lung necrosis as well as early death in mice [81] and TB reactivation in humans [82]. Interesting still, good clinical responses to anti tuberculosis therapy (ATT) is associated with reduction in TNF-α levels [73]. In patients treated with anti-TNF-α who eventually develop TB, sudden discontinuation of anti-TNF-α therapy may be associated with severe exacerbation and further lung destruction [83]. Taken together, TNF-α is an important mediator in TB pathogenesis and its function needs to be well regulated for desirable clinical outcomes.

IL-1β has also been shown to be important in *M.tb* infection [14, 81, 84]. The mechanism of IL-1β-mediated effects in TB pathogenesis is not very clear; however signalling via toll like receptors (TLR) in conjunction with IL-1β production and activation of the adaptor molecule MyD88 [84] has been shown in TB. IL-1β is important in recruiting perivascular phagocytes and promoting inflammation and granuloma formation [85]. Mice lacking IL-1β showed inability to produce IL-12 and TNF; these mice had severe lung disease and died despite a well preserved adaptive immune response [81]. Host-directed therapy targeting IL-1β has been proposed in TB, which reflects the dual nature of this cytokine in maintaining immunological balance in disease [86].

The importance of IL-17 in TB has been noted in its ability to promote quick trafficking of memory CD4+ T cells from lymph nodes into the lungs for early *M.tb* control [87], In NHP model of human TB, animals that are able to control TB showed up regulation of IL-17 pathway genes[88]. Similarly, whole blood levels of IL-17 were lower among children with TB compared to healthy controls [89].

Pleiotropic cytokine such as IL-10 and IL-6 are important in modulating the severity of TB disease but simultaneously allow enough pro-inflammatory activity for successful TB control[64]. Elevated levels of IL-10 is documented in both lung and serum of patients with TB; however its correlation with clinical presentation or outcome is variable [90]. Levels of IL 10 were significantly higher in children with TB disease compared to healthy individuals [89]. It seems plausible that some anti-inflammatory effect of IL-10 is necessary in order to eradicate *M.tb* in the granuloma [64], since a generally requirement for pro-inflammatory activity exists[91].

IL-6 is a pleiotropic cytokine with both pro- and anti-inflammatory effects that is produced by many cell types including monocytes, macrophages, dendritic cells, mesenchymal stromal cells and fibroblasts [92, 93]. Patients with active TB have higher levels of IL- 6 compared to healthy controls, and their IL-6 quantities directly correlated with systemic disease, mycobacterial load in sputum and lung injury [80]. IL-6 signals via its receptor, IL-6R in association with gp130, both of which are expressed on T and B cells, hepatocytes and

fibroblasts among others. IL6R-bound IL-6 can also promote trans-signalling, whereby the IL-6-IL6R complex would bind to gp130 on cells to initiate signalling. IL-6 signalling induces the release of several liver proteins, T-cell differentiation, B-cell activation and antibody production, secretion of vascular endothelial growth factor (VEGF), platelet generation and collagen production among others[92].

Preclinical studies indicate that IL6 may be important for survival during TB disease. Mice deficient for IL-6 succumbed to *M.tb* but not to *M. bovis* BCG challenge [30]. In one study it was reported that *M.tb* induces IL-6 to inhibit macrophage response to IFN-γ and thereby dampen the adaptive immunity [94]. Blocking IL-6 trans-signalling did not affect protection in *M.tb*-infected mice but is associated with control of inflammation [23]. However, complete blocking the IL-6R would reactivate latent TB [83]. Thus, abrogation of cytokine production from the start cannot be an option but rather modulation of their dynamics during disease. Since *M.tb* infection presents with a wide spectrum of disease (latent vs active; early active disease vs late), cytokine secretion may vary accordingly and studies in mice can't directly be translated to humans. Further clinical investigation among humans with TB is warranted to unfold the role of IL-6, and whether anti-IL-6/IL-6R therapies may be beneficial to reduce lung damage, manage immune reconstitution inflammatory syndrome (IRIS) in patients with or without HIV and optimizing MDR/XDR-TB treatment outcomes [80, 95]. It is evident that cytokines play key role in TB pathogenesis, but their role of as markers of disease severity and/or clinical response is yet to be precisely elucidated [46, 77].

1.3 TUBERCULOSIS AND HIV/AIDS CO-INFECTION PRESENT CLINICAL MANAGEMENT CHALLENGE

The burden of TB among HIV-infected individuals is high; up to 40% of HIV-infected individuals developed TB in the SSA region [96, 97]. Despite these high TB rates, 30 – 50% of TB cases are diagnosed post mortem [97-100]. HIV-infected individuals are at an increased risk for TB, exhibiting both true relapse and re-infection presenting with mixed *M.tb* strains [101]. HIV mainly infects CD4+ T cells, dendritic cells and macrophages [102], resulting in impaired control of infections such as *M.tb* due to quantitative and qualitative dysfunction of the macrophage and CD4+ T-cell responses [16]. Chetty and colleagues demonstrated that HIV/TB co-infected individuals produce less CD4+ and CD8+ T-cell specific cytokines compared to HIV mono-infected individuals suggesting that the impaired immune function could be the cause for inability to control *M.tb* [103]. On the other hand, impaired control of *M.tb* among HIV-infected individuals, who are antiretroviral therapy (ART) naïve, has been associated with immune cell exhaustion and autophagy [104, 105]. CD4+ T cells from patients with HIV/TB have been shown to express markers of cell exhaustion (T-cell immunoglobulin and mucin domain 3 (TIM3) and programmed cell death 1 (PD-1)) and exhibited reduced control of *M.tb* infection compared to T cells from healthy controls; this phenomenon was reversed with blockade of TIM3/PD1 [104]. HIV-infected macrophages demonstrate increased IL-10 production and inhibition of apoptosis[17] which is restored with early ART [16]. Overall the

number of incident TB cases has an inverse relationship as the duration of ART increases after initial immune reconstitution phase [4, 106, 107].

Diagnosis of TB among HIV-infected individuals presents an enormous challenge to clinicians, often exhibiting atypical symptoms and negative microbiological and imaging results [37, 108]. The invention of Xpert MTB/RIF has improved TB case detection compared to smear microscopy, with detection rates increased by up 60% among patients with smear negative TB [109, 110]. Yet, about 48% of smear-negative TB/HIV patients will be missed by Xpert MTB/RIF system [110]. This unmet gap calls for urgent invention of rapid and more sensitive diagnostic protocols.

HIV and TB co-infection also presents challenges during treatment. Treatment of HIV-infected individuals with ART demands careful planning for patients with TB co-infection. Concomitant administration of ATT and ART often results in unwanted drug interactions, increased toxicity and IRIS [111, 112]. RIF, a key first line anti-TB drug, reduces serum concentration of most non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors as well protease inhibitors (PIs) [113-117]. Serum levels of nevirapine and rilpivirine are greatly reduced to sub-therapeutic levels, so the combination of any of these NNRTIs is usually avoided in combined ATT and ART [114, 117]. Another anti-retroviral drug, efavirenz is affected to a lesser extent than nevirapine, therefore dose adjustment greater than 600mg daily is not usually recommended in general practice [118-120]. Rifabutin is a rifamycin of choice for patients with TB/HIV co-infection when PIs are considered [121]; however, efavirenz is expensive and not available in low-income countries where TB/HIV burden is high. In contrast, ritonavir-boosted PI decreases rifabutin to sub-therapeutic levels, necessitating dose adjustment to 150mg daily when the combination is prescribed [121]. Availability of newer anti-TB drugs such as delamanid provides a broader avenue for choice with fewer drug interactions yet good treatment outcomes although access may be a another challenge in resource-limited areas [122].

Scale-up of ART in the SSA region has tremendously improved survival of patients with or without TB [123]. However, despite the gains, the risk of TB is highest during the immune reconstitution phase of ART usually the first three to six months of ART and decreases thereafter [106]. Multiple reasons therefore contribute to less successful treatment outcomes among TB/HIV co-infected individuals compared to individuals with TB. Therefore, HIV co-infection is often a mandatory component which needs to be accounted for in TB research and clinical practice.

1.4 ISONIAZID IN THE TREATMENT AND PREVENTION OF TUBERCULOSIS

Isoniazid is a potent anti-TB drug with early bactericidal effect [124, 125]. As a first line drug of choice, INH in combination with RIF is used in standardised regimens to shorten treatment duration of drug-sensitive TB [56]. In addition, INH is recommended as standard of care for TB prophylaxis among recent tuberculin skin test (TST) converters (with or without history of close TB contact), children who are contacts of patients with PTB or HIV infected individuals

after exclusion of active TB disease [38, 126, 127]. Indeed, INH prevention therapy (IPT) is one of the three pillars of TB prevention in addition to intensified case finding and infection control[33].

The benefit of IPT in preventing mortality and reducing TB occurrence and recurrence among HIV patients is well established in the presence or absence of ART [128-130]. However, there are some practical uncertainties that need to be sorted out: the ideal duration of IPT for HIV infected individuals, exclusion of active TB among HIV infected individuals in resource-limited settings, sub-optimal compliance to IPT treatment and possibilities of emerging resistance in the long term [131-134]. Compliance to IPT is necessary to prevent mortality [135]. Variable non-compliance rates have been shown, ranging from 2 – 36% for 6 months IPT[136-140]. Worse compliance has been reported among female sex workers, among whom IPT non completion rate was 61% [141]. These challenges therefore present impediments to the proposed benefits of continuous IPT for HIV-infected individuals [129], more so when resistance to INH has been linked to non-compliance [142].

INH resistance is the most common type of resistance among anti-TB drugs in clinical use[32, 143, 144]. Globally, INH resistance does not show a decreasing pattern like that seen among patients with MDR-TB in the United States [142]. The median global prevalence of INH resistance is 13% but rates as high as 60% have been reported in some countries [32, 143]. Mathematical models predict increased INH resistance when community-wide IPT programs are accounted for [132, 145].

There are variable treatment combinations to manage INH-resistant TB, in some settings, the treatment of choice is left to the discretion of the clinician based on drug susceptibility testing (DST)[146, 147]. The WHO recommends using the RIF, Z and E regimen for 6–9 months [127]. Treatment of patients with non-MDR INH resistance shows variable outcomes. Some reports show similar success rates compared to drug-susceptible TB [148-150], while others show less favourable outcomes [146, 147, 151]. It is therefore essential to monitor INH resistance rates so as to provide evidence for programmatic decision-making for preserving drug efficacy. Notwithstanding high resistance rates for INH, standardised regimens for treating INH mono-resistant TB need better optimisation.

1.5 HOST-DIRECTED THERAPIES IN TUBERCULOSIS

Host immune responses are immensely responsible for disease progression as well as severity of lung damage in patients with TB [12]. Modulation of the host response to *M.tb* infection and during TB disease forms the mainstay of host-directed therapies against TB [152, 153]. These approaches are designed as adjunctive therapies to current anti-TB drug regimens to reduce the extent of tissue damage and improve treatment outcomes [152-154]. Safe and efficacious interventions have been shown for drug-susceptible as well as MDR/XDR-TB [83, 154]; larger clinical trials for optimization as well as to provide newer options are needed for drug sensitive and drug resistant TB. Therefore, a further understanding of the immune

correlates of severe lung injury and poor outcomes would be important to better understand TB pathogenesis, paving way for better therapies.

1.6 TUBERCULOSIS IN TANZANIA

Tanzania is one of the high burden TB countries according to global statistics [23, 30]. TB prevalence in Tanzania is estimated to be 249 – 293 per 100 000 adults and the most affected population groups are the elderly, adult males and inhabitants of rural areas [155, 156]. The distribution of TB prevalence in Tanzania is provided in Figure 7.

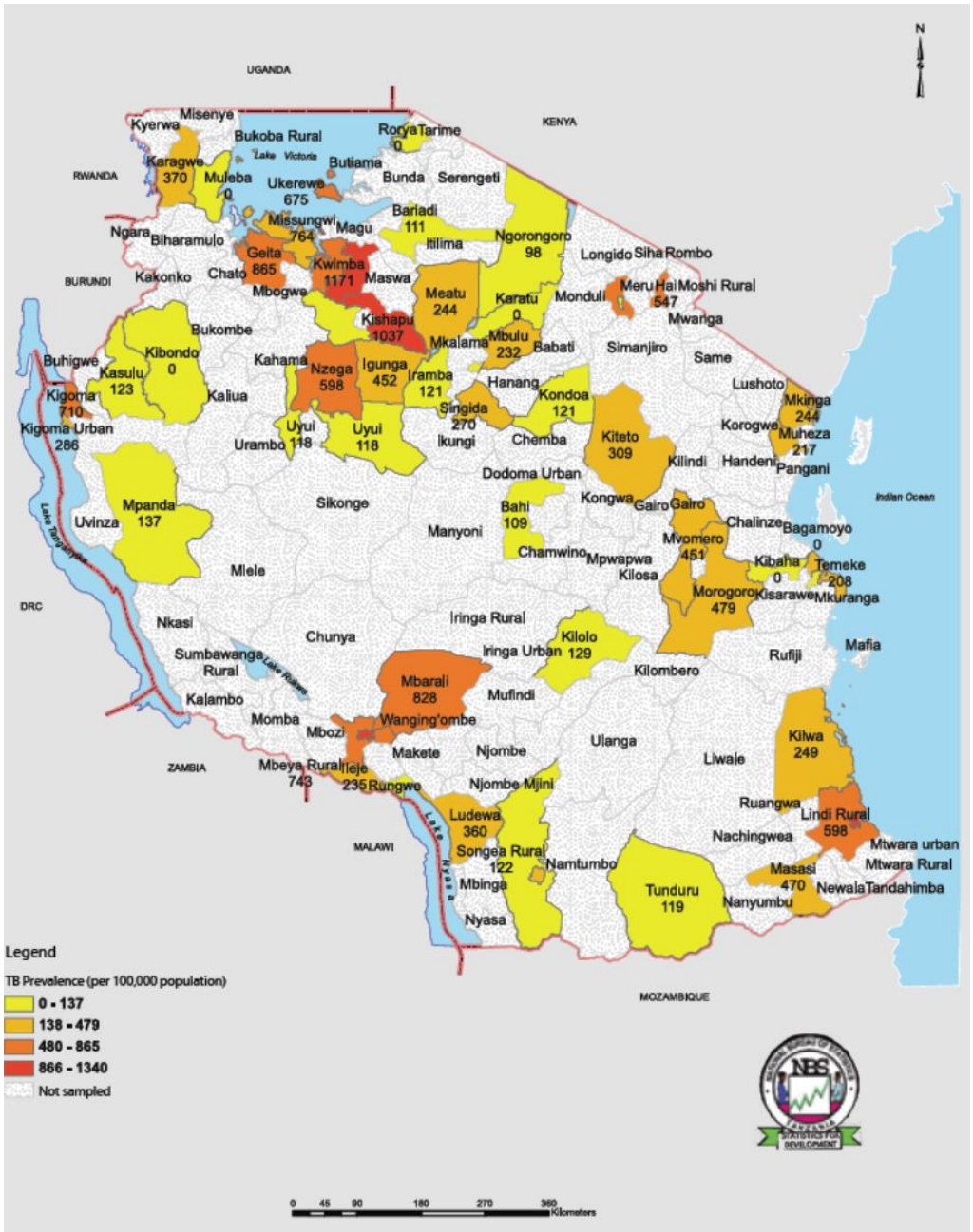


Figure 7: Prevalence of TB in Tanzania by cluster (Source: National Tuberculosis and Leprosy Program - The first National tuberculosis prevalence survey in United Republic of Tanzania final report – Ministry of Health and Social Welfare)

Country notification for all forms of TB in Tanzania for the year 2014 was 63,151 [1]. This number translates to a TB case notification of 142 per 100,000 population and 48% case

detection rate compared to the estimated prevalence [1, 29]. Increasing age is a strong correlate of TB at population level [29]; however, TB cases among the elderly are disproportionately less notified in the health system (Figure 8) [1, 155].

In Tanzania, all patients with TB are provided access to HIV testing services with the possibility to refuse. The HIV screening acceptance rate among TB patients was 88% in 2014. Among those tested for HIV, 19890 (36%) had TB/HIV co-infection and 87% of the identified TB/HIV patients started on ART within the first three months of diagnosis [1].

The national prevalence of HIV among Tanzanian adults (15 – 49 years) is 5.1%. The proportion of HIV-positivity is slightly higher in urban (7.2%) than rural (4.3%) areas, and among women (6.2%) compared to males (3.8%) [157]. Until 2016, a total of 894,356 people (children and adults) living with HIV (PLHIV) were receiving ART [158]. Due to the increased risk for TB among PLHIV, HIV clinics are important entry points to TB care and vice versa; in Tanzania. TB screening is done using a simple symptom-based tool followed by detailed investigation whenever TB is suspected [35, 36]. For TB prevention among PLHIV, IPT is provided at 300 mg dose daily for six months [36].

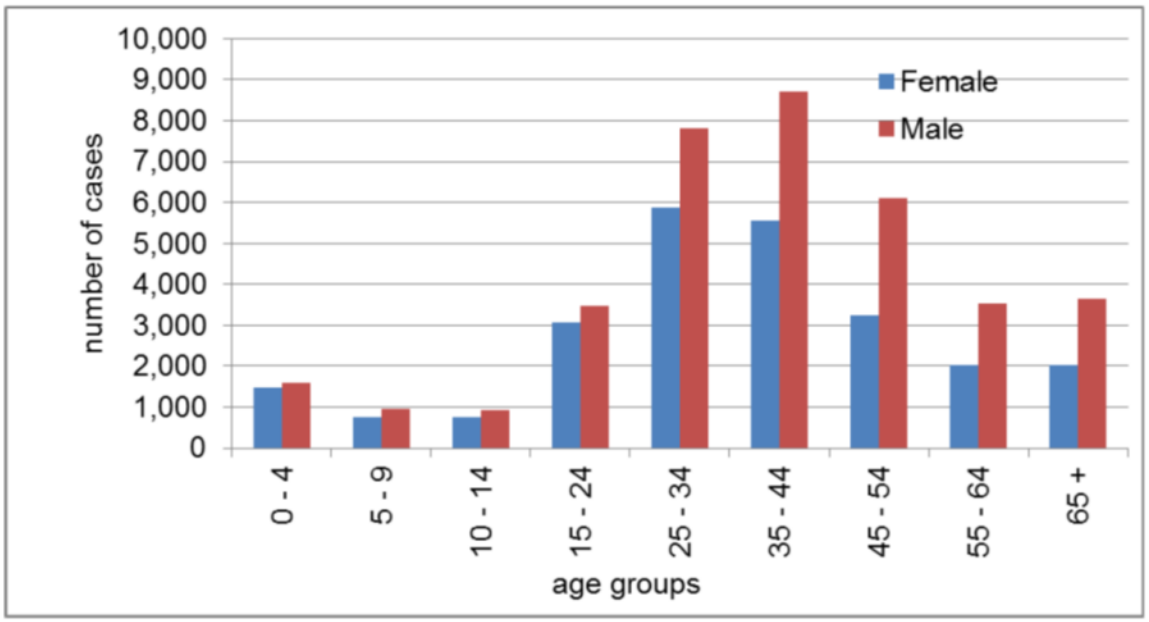


Figure 8: Age distribution of Tuberculosis notifications in Tanzania (Source: National Tuberculosis and Leprosy Program Annual report for 2014)

Drug-resistant TB is less common in Tanzania, presenting in the range of 0.2 – 1% and 4% for new and retreatment patients respectively [159]. The national health system, however, reported 142 patients with MDR-TB in 2014 [1]. Based on the national survey, about 631 new TB patients presumably have MDR-TB (1% of 63,151 patients with TB notified in 2014) [159]. Therefore, the majority of patients with MDR-TB in Tanzania may die before diagnosis. Increasing the access to rapid molecular diagnostic tools may improve detection of MDR-TB cases in the country.

Diagnosis of TB in Tanzania is mainly achieved by microscopy at primary health centres. Recent reports show that microscopy services are available in 945 facilities across the country[1]. Regional referral facilities and several district facilities provided total of 67 Gene Xpert MTB/RIF services in the country in 2015 (compared to 3,500 facilities health facilities treating patients with TB) [1, 160]. Zonal and national referral laboratories offer culture and molecular technology platforms such as LPA and Gene Xpert MTB/RIF [160].

Tanzania adheres to the WHO treatment guidelines for the clinical management TB [36, 41, 42]. Treatment is supervised via directly observed therapy (DOT). Patients have two options to choose from: facility DOT, which is observed by healthcare workers at the nearest clinic daily or community DOT, which is supervised by a pre-identified treatment supporter at the patient's residence [36, 161]. Patients on community DOT have been shown to have an ATT adherence rate of 96%, and in the initial assessment community DOT had similar cure rates but higher treatment success compared to facility DOT [162]. The treatment success for smear-positive TB in Tanzania is about 90%, and overall 3,650 (5.6%) patients with of all forms of TB notified in 2013, died at some point during the treatment period [1]. Further analysis aimed at improving treatment outcome is of prime importance to save approximately 4000 patients with TB estimated to die per year in Tanzania.

2 AIMS OF THE THESIS

The general aim of this thesis was to understand further how host immune responses and pathogen factors influence treatment outcomes of patients treated for pulmonary tuberculosis in Tanzania. We explored selected host responses (unstimulated and stimulated cytokine dynamics) as well as pathogen factors (HIV infection, antiretroviral therapy and isoniazid resistant *M.tb*) in the hope of contributing knowledge towards reducing mortality and improving quality of life of patients with pulmonary TB in Tanzania. A conceptual framework for the thesis aims is provided in figure 9.

2.1 SPECIFIC AIMS

1. To quantify the mortality burden among patients initiating first line anti tuberculosis therapy in Tanzania (Paper I).
2. To investigate the effect of timing of anti-retroviral initiation in relation to commencement of anti-tuberculosis therapy on the outcome of patients with tuberculosis in Tanzania (Paper I).
3. To compare treatment outcomes among patients with and without isoniazid resistance in the absence of MDR-TB (Paper II).
4. To explore baseline anti-EBV or anti-CMV interferon gamma reactivity as a predictor of death or survival during treatment for tuberculosis (Paper III).
5. To explore the utility of selected serum cytokine levels as biomarkers for disease severity and predictor of poor treatment outcomes for patients with tuberculosis (Paper IV).

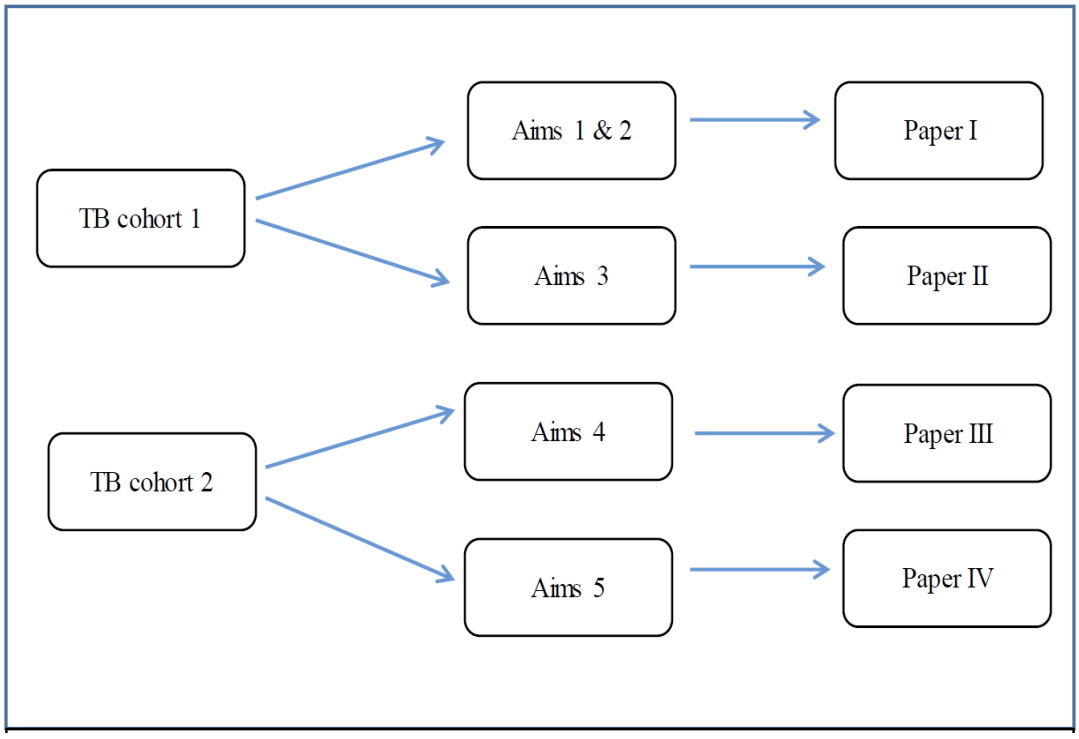


Figure 9: Conceptual framework for the stud aims

3 MATERIALS AND METHODS

Detailed descriptions of the methods used for the papers in this thesis are available in the respective papers a brief description is provided and summarised in table 1.

3.1 PATIENT POPULATION

We employed two prospective observational cohorts (cohort 1 and 2) to answer the objectives of the thesis as depicted in Figure 4. Eligibility into both studies included: Recently diagnosed pulmonary tuberculosis at one of the participating research clinics, no prior history of tuberculosis disease or treatment with isoniazid preventive therapy, intention to remain in Dar es Salaam until completion of their TB treatments.

3.1.1 TB Cohort 1 (Paper I and II)

Recruitment of patients for cohort 1 commenced in October 2010 and follow up was completed by December 2011. Patients were 15 years and older and were recruited from one of the participating tuberculosis clinics. We involved the 14 largest TB clinics from Ilala, Temeke and Kinondoni municipals in Dar es Salaam. The health facilities involved were; Amana, Mwanayamala and Temeke Regional referral hospitals; St Monica Modern hospital; Buguruni, Mnazi mmoja, Magomeni, and Sinza Health centres; Mbagala Rangitatu, Mbagala Kizuiani, Mbagala Tambuka reli, Tandale and Ukonga dispensaries; as well as the Infectious Disease Clinic (IDC) of the Muhimbili National Hospital (MNH).

3.1.2 TB Cohort 2 (Paper III and IV)

This cohort included patients aged 18 years and older recruited in Mwanayamala and Amana Hospitals and Mnazi Mmoja Health Centre between October 2013 and April 2015. We excluded patients known to have malignancies, or immunosuppressive therapy or those with end organ failure.

3.2 TUBERCULOSIS DIAGNOSIS AND TREATMENT

Diagnosis of tuberculosis was made by the attending clinicians according to the Tanzanian guidelines for diagnosis and management of tuberculosis. [36] Recruited patients were those diagnosed with pulmonary tuberculosis on the basis of sputum smear positive results. Patients provided spot and morning sputum samples and smears were examined for AFB using Ziehl–Neelsen (ZN) technique by trained laboratory technicians at participating health facilities.

All patients were managed in accordance with country guidelines [36]. Treatment consisted of daily isoniazid 5mg/kg, rifampicin 10 mg/kg, ethambutol at 15 mg/kg and pyrazinamide at 25mg/kg for 2 months (intensive phase) followed with daily 5mg/kg isoniazid and 10 mg/kg Rifampicin for subsequent four months (continuation phase). An additional month of intensive phase ATT was given in case sputum smear conversion was not attained after two

months of ATT. Thereafter, smear non-convertors were managed as having MDR-TB. Throughout duration of TB therapy, DOT was instituted.

3.3 PATIENT RECRUITMENT AND FOLLOW UP

Consenting patients entered into the study upon first prescription of anti-TB drugs. Interviews were performed by attending clinicians. In addition to routine tests, sputum and blood samples were requested at diagnosis. Patients were interviewed and had repeat tests 2 and 5 months after initiation of ATT.

3.4 LABORATORY AND CLINICAL PROCEDURES

3.4.1 HIV infection status determination

HIV infection was determined using Determine ^{TM} HIV-1/2 (Inverness Medical Japan Co. Ltd, Japan) and Uni-Gold ^{TM} HIV-1/2 (Trinity Biotech, Wicklow, Ireland) serially. Enzyme Linked Immunosorbent Assay (ELISA) was employed as third test for any discordance.

3.4.2 Sputum culture for *Mycobacteria tuberculosis (M. tb)* complex

Detection of *M.tb* was achieved in either the solid egg based Löwenstein–Jensen (LJ) or liquid media system, BACTEC Mycobacteria Growth Indicator Tube (MGIT) – (Beckton-Dickinson) according to manufacturer's instruction.

3.4.3 Drug Susceptibility Testing (DST)

Drug susceptibility testing was done using the proportion method. *M.tb* H37Rv strain was used as control. The following drug concentrations were used for DST, 0.2mg/l for isoniazid, 40 mg/L for rifampicin, 4 mg/L for streptomycin and 2 mg/L for ethambutol.

3.4.4 Whole Blood Assay (WBA)

Two millilitres of diluted whole blood was added into 96-well sterile culture plates pre-coated with: TB antigens: ESAT-6, Ag85A, Rv0447c, Rv2957, Rv2958c; common viral antigens: EBNA1, CMVpp65, HSV-1, H5N1, H1N1, HIV antigens: HIV env, HIV gag; and controls negative (RPMI) and positive (PHA). The plates were incubated 37°C in a 5% CO_2 incubator for 7 days when supernatant was transferred into non-sterile 96-well plates and frozen at -80 °C for IFN-γ Enzyme Linked Immunosorbent Assay (ELISA) determination later.

3.4.5 Cytokine determination by Enzyme Linked Immunosorbent Assay

Serum concentrations of unstimulated IFN-γ, TNF-α, IL-2, IL-6, IL-10, IL-17A and IL-21; as well as WBA-supernatant concentration of IFN-γ were determined using ELISA kits (Mabtech, Stockholm, Sweden) according to manufacturer's instructions. Standard curve was

plotted and was then used to determine concentration of the cytokines based on Optical Density (OD) value.

3.4.6 Chest radiographs

Postero-anterior chest radiographs were done at entry and upon treatment completion for a subset of the study population in cohort 2. Radiographs were assigned scores using a validated tool by Ralph A and colleagues [47]. A minimum score of zero was given where there were no radiographic changes while highest score of 100 was assigned when all zones in both lungs were affected homogenously. Presence of cavity regardless of size and number attracted an additional the score of 40. Consequently, the maximum score for any radiograph in the presence of cavities was 140.

Definitions of chest injury:

Severe lung damage at TB diagnosis: Chest x-ray (CXR) score equal to or above 80 (75th percentile) at TB diagnosis.

Severe lung damage at TB treatment completion*:* CXR score equal to or above score of 50 (75th percentile) at end of ATT.

3.5 DATA PROCESSING

Case record forms were used to capture demographic, clinical and laboratory data which was subsequently transformed to electronic data using Epi info 6 data capture screen. Analysis was either done by either SAS version 9.3 (paper I) or Statistical Program for Social Sciences (SPSS) version 23 (Paper II – IV) as summarised in table 1.

3.6 ETHICAL CONSIDERATIONS

This doctoral project was based on observation study involving human subjects. The project involved; patient interview, taking extra samples, access to the patients' clinical information relating to their treatment as well as outcome.

Ethical approval was obtained for both cohorts 1 and 2 of this thesis. Patients' autonomy, rights and safety were always safeguarded. Eligible patients received information sheets with study details. Patients were guaranteed of appropriate health care even if they did not consent to participate in the study. The patient's relative or another health care worker unrelated to the study assisted patients unable to read and/or write. Parents or guardian provided consent on behalf of minors (patients between 15 – 17 years). Written consent was provided by signing on special consent forms. Patients were interviewed privately; notes were stored in locked cabinets; and the subsequently created electronic database was stored in password protected computers.

Table 1: Summary of materials and methods for studies used in this thesis

Aim (s)	**Study Design & Study population(n = sample size)**	**Main outcome of interest**	**Main exposure of interest/ Laboratory methods/read- out**	**Main statistical analysis methods**
1/2	Prospective cohort of Tuberculosis patients (n = 1696)	All cause mortality	HIV Anti-retroviral therapy (ART)	Cox-proportional hazards model
3	Prospective cohort of Tuberculosis patients without multi-drug resistance TB (n = 861)	Unfavorable treatment outcome (Death, treatment failure and loss to follow up)	Isoniazid resistance by MGIT and LJ cultures	Logistic regression
4	Prospective cohort of Tuberculosis patients (n = 234)	Death or survival	Anti-gamma interferon level (pg/ml) to EBV from supernatant on whole blood Assay Anti-gamma interferon level (pg/ml) to CMV from supernatant on whole blood Assay	Mann-Whitney U test
5	Prospective cohort of Tuberculosis patients (n = 234)	Death or survival Severe lung injury at baseline (high x-ray score >80) Severe lung damage post treatment (score > 50)	Unstimulated serum cytokines by Sandwich ELISA on serum IFN-γ, TNF-α, IL-6,IL-21, IL-17 , IL-10	Logistic regression General linear model for repeated measure analysis

4 RESULTS AND DISCUSSION

4.1 BURDEN OF TUBERCULOSIS MORTALITY

Paper I: Int J Infect Dis. 2017 Mar; 56:39-44

A total of 1696 out of 1805 patients with tuberculosis were included in this paper, among them 58 (3.4%) deaths occurred within six months of ATT. We excluded 109 patients because they had missing information such as HIV, outcome and age. The excluded patients were more likely to be males and had history of illicit drugs use; factors linked with increased mortality risk among patients on ATT [163]. Due to this potential bias therefore, we might have underestimated mortality. In the worst case scenario, if all 109 excluded patients are considered dead, then, mortality would be 9.3%. Thus, it is very likely that the true burden of mortality among patients with TB in our study would be between 3.4 – 9.3%.

The mortality risk was higher among TB/HIV co-infected individuals (41/514; 8.0%) as compared with TB mono-infected patients (17/1182; 1.4%), a phenomenon reported among patients with HIV especially in advanced disease [96, 164, 165]. Most probably as a consequence of multiple factors; related to immune suppression, [16] immune reconstitution or, not mutually exclusive, competing drug toxicity [111, 166].

The median time to death in our cohort was 46 days and at the end of intensive phase of ATT two thirds of all the deaths had occurred. This accelerated initial mortality warrants further investigation in relation to exaggerated inflammation. The initial phase of ATT is associated with exponential killing of *M.tb* resulting in a surge of *M.tb* antigens accessible to the immune system [9]. Worsening of clinical features following effective ATT; also known as *paradoxical TB,* has been described among HIV infected and uninfected individuals and might be related to exaggerated inflammatory response [166, 167]. On the other hand, early deaths may signify advanced disease owing TB diagnostic challenges [37]. It could be hypothesized that, late presentation is associated with large TB antigen burden or a mere host exaggerated inflammatory response but this remains unanswered by this study and calls for further studies.

4.2 TUBERCULOSIS MORTALITY PATTERN IN RELATION TO ANTI-RETROVIRAL THERAPY INITIATION

Paper I: Int J Infect Dis. 2017 Mar; 56:39-44

It was apparent that TB/HIV co-infected patients (n = 514) had higher mortality risk thus warranting a much deeper scrutiny in relation to HIV treatment. As shown on table 2; patients who initiated ART 90 days before starting ATT or within first 14 days of ATT had highest mortality (10%), whereas lowest mortality was observed among TB/HIV who had initiated ART after 14 days of ATT (5.0%) (Table 2).

Table 2: Crude mortality pattern in relation to HIV infection and anti-retroviral therapy (Nagu, IJID 2017)

	No. of deaths	**No. at risk**	**Crude mortality rate (%)**	**Median days to death (IQR)**
TB+/HIV	17	1182	1.44	46 (32, 62)
TB+/HIV+; ART >90 days prior to ATT	5	49	10.20	79 (33, 96)
TB+/HIV+; ART ≤ 90 days prior to ATT or ≤14 days after ATT	4	39	10.26	56 (27.5, 72)
TB+/HIV+; ART >14 days after ATT	7	141	4.96	52 (45, 89)
TB+/HIV+; No ART	25	285	8.77	37 (26, 59)
Total	58	1696	3.42	46 (30, 72)

IQR = Inter Quartile Range, TB = Tuberculosis, ATT = Anti Tuberculosis Therapy

In this cohort, less than half of TB/HIV patients (229/514) were initiated on ART during the follow up period. According to Tanzanian guidelines at the time, all HIV co-infected TB patients had to be initiated on ART, preferably after the second week of ATT or within the first two weeks in case of severe immunodeficiency (CD4+ T lymphocytes < 50 cells/μL) [36]. The low proportion of HIV infected patients on ART is probably a reflection of a cascade of events including late HIV diagnosis, patients' refusing to take ART, poor referral and lack of integration of HIV and TB services at the time. Integration of HIV and TB services has lately improved ART uptake to 87% but mortality remains at 6% [1]. Since, TB clinics serve as an important entry point to HIV care and treatment, attempts to harness integrated TB and HIV services for appropriate and timely interventions would greatly improve treatment outcomes.

Using multivariate analysis we compared mortality in relation to ART and ATT among TB/HIV co-infected patients, while TB patients without HIV co-infection was the reference group. Mortality risk was lowest (three fold) when ART was initiated more than 14 days after ATT and highest when ART was initiated before or within the first14 of ATT. Persistent high mortality among TB/HIV patients despite good HIV virological control is yet to be thoroughly understood among patients on ART. Apparently there is heterogeneity in regaining overall immune function after ART initiation despite viral suppression which may be associated with lack of TB control and/or mortality [165]. It suffices to say at this point that,

ART and ATT initiation by themselves are insufficient to avert some deaths among TB/HIV patients on ART. It is thus of interest to further investigate the functional immune reconstitution in relation to clinical recovery and/or possibility to intervene with host directed therapies to reduce these unaccounted mortalities.

Patients with advanced HIV particularly those with TB/HIV need vigilant follow up of clinical, virological and immunological parameters; and prompt action should be taken when required[165]. TB/HIV patients, who had initiated ART more than three months prior to ATT in our cohort, had low median CD4+ T lymphocyte count (263 cells/μL) and median BMI of 18.8kg/m^2. These clinical parameters are associated with opportunistic diseases and thus may be synonymous with a failing ART regimen. We did not quantify HIV viremia, neither did we study ART resistance nor the functional CD4+ T cell immune recovery in relation patients who died. This might be regarded as an area to improve the quality our study in order to add further knowledge for even better TB/HIV management. We discuss inflammation in relation to mortality in papers III and IV.

Given the overall high initial mortality, it is plausible to hypothesize that the individuals who died before ART was initiated had been sicker and therefore incomparable to those in the ART group. This hypothesis may not likely in our cohort as patients had similar median CD4+T cell count and the body mass index (BMI) as seen in Table 3

Table 3: Distribution of median CD4+ T lymphocyte counts and body mass index by anti-retroviral therapy among TB/HIV co-infected patients (BMI = Body mass index; IQR = interquartile range) (Nagu, IJID 2017)

	Median CD4+ (IQR)	**Median BMI kg/m^2**
TB+/HIV+; ART > 90 days prior to ATT	263 (181, 422)	18.8 (17.0 -21.5)
TB+/HIV+; ART ≤ 90 days prior to ATT or ≤ 14 days after ATT	168 (79, 221)	17.4 (15.4, 19.6)
TB+/HIV+; ART >14 days after ATT	151 (69, 220)	18.8 (16.9, 21.2)
TB+/HIV+; No ART	331 (158, 471)	18.1 (16.5, 20.4)

IQR = Inter Quartile Range, TB = Tuberculosis, ATT = Anti Tuberculosis Therapy

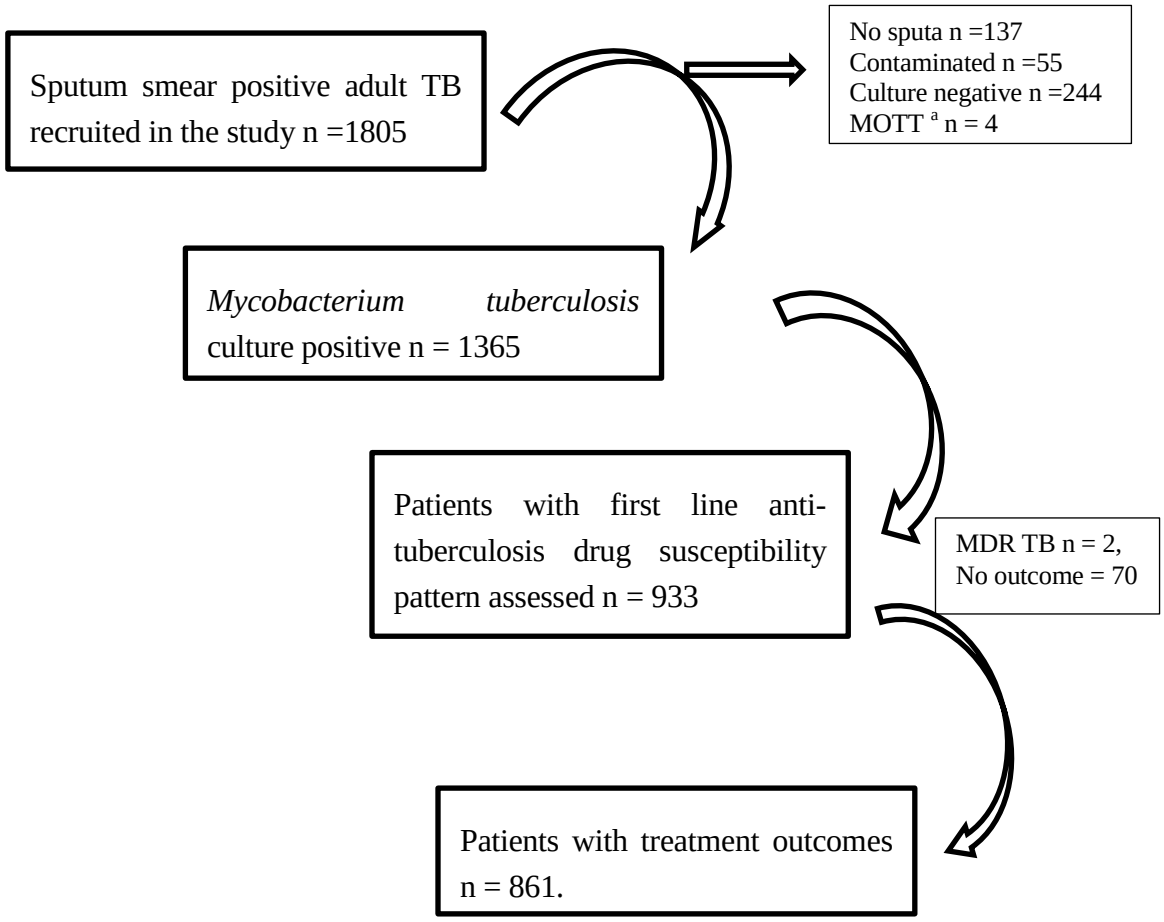
There were yet HIV infected patients who developed TB following ART in our cohort 1 (Table 2). Unmasking TB is a term coined to TB occurring shortly after initiation of ART usually in the first three months of ART [107, 168]. The increased rates of TB soon after ART are probably a result of TB missed during clinical evaluation prior to initiation of ART a fact that underscores need for intensified TB screening including use of culture and gene Xpert MTB/RIF pre-ART[108]. Unfolding TB might explain the occurrence of TB among the patients who had initiated ART around 90 days prior to or immediately after ATT (within 14 days) (Table 2).

The cohort was almost entirely composed of young to middle aged patients, (91% were ≤ 50 years; and two thirds were males (67%), a finding that is in keeping with many parts of developing countries with high burden of tuberculosis [23]. Two important things to consider include i) increased propensity of such patients to infect their family members as well as colleagues at work places even before diagnosis, as determined by mycobacterial load in the sputum [9]. and ii) reduced productivity due to prolonged illness related sick leave leading to poverty and malnutrition, a vicious circle of tuberculosis. Indeed, poverty and tuberculosis are cousins who must be addressed hand in hand to break the circle [10]. It is through improved living conditions that TB was significantly reduced in high income countries.

4.3 RESISTANCE TO ISONIAZID AND TUBERCULOSIS TREATMENT OUTCOMES

Paper II: J Antimicrob Chemother. 2017 Mar 1;72 (3):876-881

This study included 861 TB-culture positive patients who had both TB treatment outcome and anti-TB drug susceptibility reported, without dual resistance to isoniazid and rifampicin (MDR) as shown in figure 10.



[a]MOTT- Mycobacteria Other Than Tuberculosis

Figure 10: Patient flow chart (Nagu, JAC 2016)

Isoniazid resistance was present in among 23 (2.7%) patients, in combinations with streptomycin 2/23(8.7%) or ethambutol resistance 2/23 (8.7%). Patients with rifampicin/isoniazid co-resistance were excluded prior. During six months of follow up, 25 (2.9%) patients died, 10 (1.2%) patients failed treatment and 29 (3.4%) patients were lost from follow-up at the clinics.

A total of 18/23 (78.3%) patients with resistance to isoniazid had successful treatment outcomes compared to 779/838 (93.0%) patients with preserved isoniazid susceptibility.

At multivariate analysis (Table 4) patients with isoniazid resistance isolates were six times more likely to have poor treatment outcome (RR 6.0; 95%CI 1.9 – 18.7; P < 0.01). In addition, HIV infection with (RR 2.3; 95%CI 1.0 – 5.2; P = 0.05) or without ART (RR 3.1; 95% CI 1.5 – 6.2; P < 0.01) was associated with increased risk for unfavorable treatment outcomes. (Table4)

Table 4: Factors associated with unfavorable treatment outcomes among tuberculosis patients initiating first line treatment in Tanzania (Nagu, JAC 2016)

	Univariate				Multivariate[a]			
	RR	95% C.I. for RR		P	RR	95% C.I. for RR		P
		Lower	Upper			Lower	Upper	
Age (years)	1.0	1.0	1.0	0.83	1.0	1.0	1.0	0.85
Male sex	1.4	0.8	2.4	0.27	1.6	0.8	3.3	0.16
Isoniazid resistance	3.7	1.3	10.2	0.01	6.0	1.9	18.7	**<0.01**
HIV status				<0.01				**<0.01**
HIV+/ART+	2.0	1.0	4.1		2.3	1.0	5.2	
HIV+/ART-	2.8	1.5	5.1		3.1	1.5	6.2	
Primary school or below	1.8	0.8	3.6	0.13	1.2	0.6	2.6	0.60
Smoking Status								
Never smokers				0.10				0.78
Past smokers	1.8	1.0	3.1		0.9	0.4	2.0	
Current smokers	0.6	0.2	2.7		0.6	0.1	2.6	
BMI (kg/m^2)	0.9	0.9	1.0	0.19	1.0	0.9	1.0	0.32

ART = Antiretroviral Therapy, BMI = Body Mass Index RR = Relative Risk CI = Confidence Interval)

Initial primary isoniazid resistance although has relatively low prevalence, is associated with poor outcome. These patients harboring resistant *M.tb* would otherwise go unnoticed or discovered late during treatment since anti-TB drug susceptibility testing is reserved to those failing first regimen or patients with a relapsed TB in Tanzania. Phenotypic assessment of drug resistance is resource and technically demanding. In many setting Gene Xpert MTB/RIF has simplified and improved detection of both TB and rifampicin resistance [109, 110]. However, given the fact that resistance to isoniazid far more common than rifampicin [143, 159], it is likely that some cases will be missed by Gene Xpert MTB/RIF system. Patients with undetected INH resistant isolates are likely progress to MDR and/or transmit the resistant strain to close contact, consequently defeating one of the three strong pillars of the END TB strategy [10, 33]. Accordingly, effective mechanisms to identify and appropriate manage patients with INH resistant *M.tb* are warranted. In addition, simple treatment regimens for INH resistant TB ought to be standardized to enable primary medical personnel to easily administer as it is with management of drug sensitive and MDR TB [42, 56].

4.4 ANTI-EBV OR ANTI-CMV INTERFERON GAMMA REACTIVITY AND TUBERCULOSIS TREATMENT OUTCOMES

Paper III: Int J Infect Dis. 2017 Mar; 56:136-139

We recruited 274 patients in this cohort; forty (40) patients were excluded in this report as shown in figure 11 below.

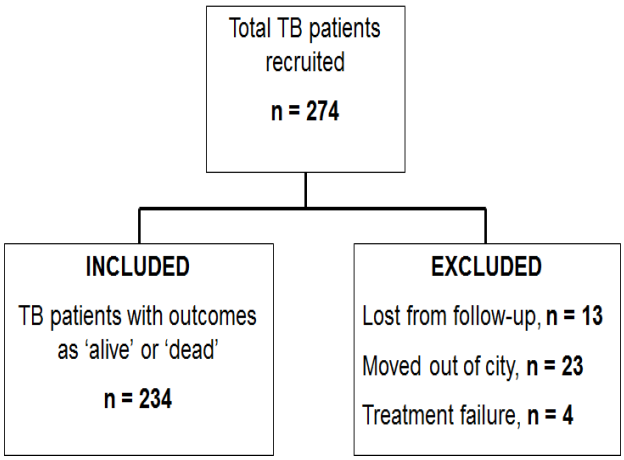


Figure 11: Patients flow chart

A total of 21 deaths occurred during the course of follow up while 213 patients were successfully treated. As in cohort 1, we noted in this cohort as well, an accelerated initial mortality since 72% of the patients died within the first three months of ATT.

Patients with tuberculosis who died, exhibited significantly lower median IFN-γ responses to ESAT-6 (0.043), CMV pp65 (p= 0.035) and EBNA1 (p = 0.006) in the whole blood assay at the time of diagnosis (Figure 12). There was no statistically significant difference in IFN-γ responses against the following antigens: Rv2958c, Rv0447c, PPD, Ag85A, herpes simplex virus 1 (HSV-1) glycoprotein and HIV env. (Figure 12)

The higher IFN-γ responses to ESAT-6 among survivors of TB, is a reflection of good *M.tb* antigen specific T cell response against *M.tb*. Coupled with similar pattern specific T cell responses against tuberculosis (ESAT-6), responses against EBV and CMV antigens may signal a correlation with good immune response against TB. Whether it is a general reflection of a good immune system or rather that a prior sensitization with EBV and CMV antigens prepared the immune system to be more alert for the mycobacterial antigen is yet to be ascertained. It has been reported that non-specific sensitization of alveolar macrophages was an important factor for their activation to achieve better control of TB infection [169]. Prospective experimental models with CMV or EBV prior to TB infection could help decipher this theory. CMV infections has been shown to drive TB immune response towards memory and terminally differentiated effector T cells [170], cells which play an important role in controlling TB. EBV also induces potent Th1 responses in EBV-positive individuals. Demonstrating CMV/EBV DNA in these patients would be helpful in

interpreting these results in consideration of prior or acute infection which would differ in their immune activation states [15, 170].

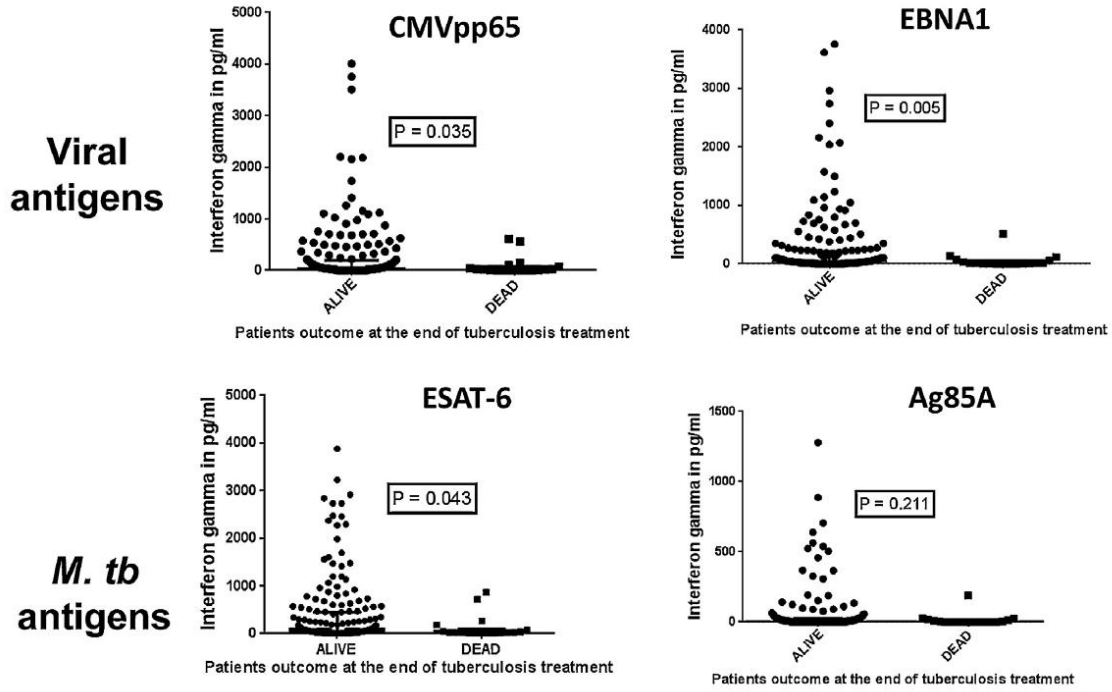


Figure 12: IFN-γ responses to viral and mycobacterial antigens in whole blood assay at the time of TB diagnosis in relation to survival during treatment with anti tuberculosis therapy (Nagu, IJID 2017)

4.5 COULD CYTOKINES PROVIDE A RATIONAL BIOMAKER FOR MORTALITY AND LUNG DAMAGE RISK STRATIFICATION

Paper IV: Manuscript

At the time of TB diagnosis we found in serum from 234 patients with pulmonary tuberculosis significantly increased amounts of IL6 (median: 3863 pg/ml) compared to the amounts in serum from seven healthy controls (11pg/ml). In addition IFN-γ, TNF-α and IL-10 levels were also upregulated in patients with TB compared to the healthy controls but the difference were not as profound as it were with IL-6. Patients who died had significantly lower median serum levels of IL-6 at diagnosis compared to those who survived and were cured.

The median CXR score at TB diagnosis was 60. Compared to TB survivors, patients who died had lower median CXR score at diagnosis. The CXR scores tended to decrease at the end of treatment among TB survivors; and in addition we observed a significant linear correlation between diagnosis and end of treatment CXR scores ($r = 0.44$, $P < 0.0001$). In summary, patients with more lung damage at diagnosis tended to have more residual damage at the end of treatment. As much as this seems intuitive, the x ray scores could be utilized in stratifying those who have high risk of severe lung damage for an intervention that might reduce this eventuality. Adjuvant treatment with cytokines and particularly IL6 (or anti-IL-6, dependent on the timing of treatment), is proposed as one possible clinically relevant area for further research as discussed later in this chapter.

Increasing serum IL-6 and IFN-γ concentrations were significantly associated with reduced mortality risk. IL-6 levels observed at diagnosis may protect against death, patients who survived TB had significantly increased lung damage at the end of ATT. This observation is in line with previous findings in sera of patients with TB from Bangladesh [171] and South Africa[172].

As previously reported, IL-6 is important in clearing large virulent mycobacterial load through IFN-γ induction. IL-6-deficient mice were shown to produce significantly lower IFN-γ and elevated IL-4 levels in response to *M .tb* challenge, and succumbed to infection within 50 days. Conversely, IL-6-deficient mice infected with the *M. bovis* BCG vaccine strain were able to control the bacterial burden almost as efficiently as wild type mice[173] which may probably signify the importance of IL-6 in *M.tb* but not *M.bovis BCG* strains.

At TB diagnosis, high levels of circulating IL-6 associates with extensive lung tissue destruction among patients with TB, based on CXR findings. This is in keeping with previous findings in patients with pulmonary TB: i) high levels of circulating IL-6 in TB patients with or without chronic obstructive pulmonary disease (COPD), in conjunction with impaired pulmonary function (marked by reduced FEV1 values) [174], and ii) increased IL-6 levels in bronchoalveolar lavage fluid, with extensive computed tomography findings in the lungs [175].

Additionally, the IL-6 levels in serum/plasma at the end of the treatment are not much lower than after two months of ATT. In support of our observations, several clinical studies have shown that IL-6 is upregulated in patients with TB undergoing therapy[176-178]. Thus, it appears that the continuation phase of ATT (last four months, daily INH and RIF only), while contributing to microbiological cure, does not promote pulmonary tissue healing in patients with TB who sustained extensive lung pathology, nor does it help reduce the high IL-6 levels present before and during ATT.

Inflammation-induced tissue damage during ATT leads to a decline in life quality in the long term, although the patient effectively clears *M.tb* infection of the lungs [2, 179]. The link between pulmonary TB and impaired lung function despite completion of therapy has also been reported for patients in South Africa with drug-sensitive[180] or multidrug-resistant TB[181]. As a consequence, these individuals are denied of a normal lifestyle thereafter, which affects their contribution to the workforce and directly affecting the country's socioeconomic development. This scenario calls for host-directed therapy (HDT) targeting overt inflammation in addition to conventional antibiotics against *M.tb* not only to prevent early mortality but also to improve life quality of individuals surviving pulmonary TB.

Anti-TNF-α therapy with adalimumab (given adjunctively to standard ATT) during severe pulmonary TB has been shown to be life-saving in severe pulmonary TB [83]. The findings in the current study lay the foundation for future studies of HDT targeting exaggerated levels of circulating IL-6 in pulmonary TB. Monoclonal antibodies targeting IL-6 (siltuximab) and its receptor (tocilizumab) are in the clinic, approved for treating arthritis and Castleman's disease [182]. Further clinical trials are underway to test their therapeutic efficacy in other indications i.e. diabetes mellitus, pancreatic, lung, ovarian cancers, myeloma[183, 184]. One preclinical study in a mouse model of TB showed that blocking IL-6R with a monoclonal antibody allows for reduced lung inflammation, improved control of lung *M. tb* burden and prolonged survival of the animals [185]. However, in the preclinical study, the animals were given the anti-IL-6R monoclonal antibody prior to *M. tb* challenge. Since IL-6 production by human monocytes is crucial for the early control of *M. tb* replication [186], and is required for initiation of adaptive immune responses to *M. tb* antigens [187], we propose that HDT directed at reducing circulating levels of IL-6 in patients with pulmonary TB would be best administered after the first two months of intensive TB treatment. The efficacy of this intervention is best studied in line with reduced inflammatory markers in serum i.e. unbound IL-6, C-reactive protein (CRP), tissue scarring (CXR scores) and restoration of lung parenchyma and pulmonary function (spirometry).

PTB is mainly local disease whose pathogenesis is complex and resulting from many factors including innate and adaptive immunity cells, cytokine and chemokines. Picture we deduce from blood therefore, is but a small part of the whole network responsible for TB

pathogenesis and control. Yet a very important suggestion that we deduce is that intervening with cytokine adjuvant therapy in addition to ATT may modulate this network towards better TB control in reducing local inflammation in the lung.

5 CONCLUSIONS AND RECOMMENDATIONS

In Tanzania, mortality among newly diagnosed TB patients during the course of treatment with first-line regimen is between 3.4 – 9.3%. This mortality is higher among patients with TB/HIV and was lowest when ART was instituted after 14 days of ATT. These findings have a great use in real life clinical practice in Tanzania and other similar setting of high rates of TB and HIV co-infections. Tuberculosis clinics have shown to be a major entry point for HIV care since the majority of our patients were first time diagnosed at TB clinics. In current view of "HIV test and treat strategy", where everyone found to be infected with HIV will be treated promptly, it is prudent to wait until the third week of ATT. For patients who have been on ART before TB was diagnosed, vigilant monitoring of clinical response and timely intervention is essential.

Background isoniazid resistance is not uncommon and has proved to cause un- favorable treatment outcomes in at least 21% of patients with resistance. Current available *M.tb* culture and DST techniques are demanding in terms of infrastructure and skills for country like Tanzania. Research for point of care, technology friendly machines for early detection of isoniazid and/or rifampicin resistance, need to be prioritized and accessed in routine TB care in order to improve treatment outcomes.

Cytokine responses are essential to successful TB treatment as well as reduction of severe lung inflammation. Whereas serum level of IL-6 are comparatively higher than of other cytokines in sera of patients with pulmonary tuberculosis and are predictive of survival; they could also be a cause of severe pulmonary damage in these patients. Adjunct therapy with IL-6 may serve as possible target in reducing lung damage following TB disease. Timing is of essence: and it may very well be that anti-IL-6 treatment (during the consolidation phase of the TB disease, associated with lung damage), could be a clinically viable option; similarly to the 'double-edged sword' described for anti-TNF alpha blockage during different phases of TB disease development [83]

More studies on inflammation and lung damage, including linking them to lung function tests; longer term cytokine follow for chronic lung inflammation including (COPD); Trials of IL 6 for TB patients as adjuvant therapy especially during the continuation phase of ATT are really essential. Further gauging anti-CMV/EBV responses in *M.tb* disease in patients with known CMV/EBV viremia would certainly add value.

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